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Ectopical expression of FABP4 gene can induce bovine muscle-derived stem cells adipogenesis

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ABSTRACT

Fatty acid binding protein 4 (FABP4) plays a key role in Fatty acid catabolism in mammals. Findings from our previous studies have indicated that FABP4 neither affect the differentiation of bovine preadipocytes nor does it change the expression of upstream genes. To investigate whether ectopically expressed FABP4 can induces Muscle-Derived Stem Cells (MDSCs) lipid synthesis and understand the regulatory mechanism behind it. In this study, adenoviruses infection is achieved to ectopically expressed FABP4 in bovine MDSCs, RNA-seq analyses at the very early stages of induction were performed to reveal gene expression level changes during MDSCs transdifferentiation. Results showed FABP4 can induce bovine Muscle-Derived Stem Cells transdifferentiation into adipocyte-like cells, 23 genes' expression levels changed after 24 h inducing although there is no significant change in cell phenotypes. Along with induction time, more differently expressed genes (256 genes changes after 48 h induction) were screened out. These genes should be at the downstream of signal pathways and be regulated by the 23 genes identified before. Our findings may provide a unique new model for studying the molecular control of cattle cross-talk between adipose and skeletal muscle.

1. Introduction

The cross-talk between adipose and skeletal muscle is complex. Better understanding of this communication will give rise to strategies to improve human health as well as for sustainable production of high quality meat. In farm animals, meat quality is closely related to the intramuscular fat (IMF) content [1,2]. Composition as well as distribution and amount of IMF have been the targets because these parameters equally influence the eating quality of beef.

It is reported that the hyperplasia of adipocytes plays an important role in intramuscular fat content during growth of muscle in cattle, and the increase of adipose tissue mass can be due to the increase of adipocyte numbers and size. However, the formation of cattle intramuscular fat and differentiation of intramuscular adipocyte are often restricted and limited [3,4]. There are increasing evidences to support that muscle-derived stem cells (MDSCs) can be used for tissue engineering and regenerative therapy [5]. MDSCs have the ability to differentiate into multiple types of cells, including myogenic, hematopoietic, osteogenic, adipogenic, and chondrogenic-like cells [6]. In the developmental process of mammal, there is an inverse correlation between skeletal muscle and adipose tissue development. When the muscle atrophy happens, the growth of adipose tissue replaced the growth of the muscle tissue. It is reported that in mice with interrupted bHLH protein, fat cells is considered to be a substitute for muscle [7]. The diverse ability of MDSCs as well as the potential alternation between skeletal muscle and adipose tissue led us to address the question of whether MDSCs could be transdifferentiated to an adipocyte cell phenotype in cattle. The current study is carried out with aim to elucidate the molecular mechanisms that regulate IMF development in farm animal species.

Adipocyte differentiation is a complex process which is regulated by a cascade of transcription factors [8]. Recent researches showed that over-expression of adipogenic transcription factors C/EBPα(CCAAT/enhancer-binding protein α), PPARγ(peroxisome proliferator-activated receptorγ) and SREBP-1 (sterol regulatory

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element binding protein1) can induce transdifferentiation of different cell types into adipocyte-like cells [9,10]. However, the potential of FABP4 (also known as aP2) to induce transdifferentiation of one cell types into another cell type remain unexplored. This protein has high affinity to the fatty acids, participated in the transshipment of long chain fatty acids and expressed higher in mature adipocytes than other cells [11,12]. Recently, FABP4 was also suggested to be significantly associated with marbling effect and subcutaneous fat depth through SNP analysis in bovine [13]. FABP4 has been reported to be constitutively expressed in adipocytes and macrophages cell types. Additionally, it was also found in coronary endothelial cells, trophoblasts and epithelial cells, suggesting additional biological roles for FABP4 [14]. Data from our previous studies suggested that FABP4 may play a vital role in the complicated gene network of adipocyte differentiation. Since FABP4 is one of the downstream genes of PPARγ signaling pathway, it can positively regulate gene expressions of adiponectin and leptin [15]. Some of the key questions we wanted to address in current study are if FABP4 was ectopically expressed in skeletal muscle, can it induce lipid synthesis in bovine MDSCs? More importantly, we also sought to identify the core genes that are involved in the transdifferentiation at the very early stage of inducing.

2. Materials and methods

2.1. Isolation, culture and immunocytochemistry of cattle MDSCs

Primary muscle cultures were prepared from newborn fetal cattle. The muscle cells were isolated from the Longissimus muscle. As previously described, different populations of MDSCs were isolated based on their adhesion characteristics [5]. MDSCs cultured in culture medium (containing 20% FBS, 10%HS, 100 UI/mL penicillin and 100 g/mL streptomycin).

The sixth population of mixed muscle cells can be used and taken passage when reach 90% confluence. Immunocytochemistry staining were constructed as previously described. Cells were plated in a 6-well culture dish and after rinsing with PBS three times. The primary antibodies were diluted in PBS which contain 3% BSA as follows: mouse anti-desmin (1:50; Santa cruz, America), goat anti−Pax3/Pax7 (1:50; Santa cruz, America), goat anti-CD34 (1:100; Santa cruz, America), goat anti-Sca-1 (1:100; Santa cruz, America). Appropriate biotinylated secondary antibodies (Santa cruz, America) for non-biotinylated primary antibodies were applied. The nuclei of cells were stained by 4′6-diaminido-2-phenylindole (DAPI; Sigma-Aldrich). The cells were visualized by fluoroscopy, and the percentage of positive cells was calculated by counting positively stained cells under Olympus 70 x fluorescence microscope (Olympus, Japan) [16].

2.2. Transdifferentiation of MDSCs to adipocyte-like cells

Adenovirus vectors were constructed as previous described. Fragments containing complete CDS areas of cattle FABP4 gene were ligated with AdEasy expression vector, and the subsequent FABP4-adenoviruses (Ad-FABP4) were produced in HEK 293A cell line [15]. Cell culture medium was changed every 3 days until cells reached confluence. Cells of 100% confluence were first starved for 9 h and then infected with Ad-FABP4 and Ad-NC (none gene-adenoviruses), respectively. Normal cells served as control. The morphological changes of the cells were studied using a light microscopy.

2.3. Cellular lipid droplet staining and quantification of lipid accumulation

The Oil Red O (Sigma) staining method was used to detect the accumulated cellular lipid droplets that were typically observed in mature adipocytes. Lipid accumulation was measured by Oil Red O staining extraction assay at 0 h, 24 h, 48 h and 96 h after infection. Cultured cells plated on 6-well plates and washed twice with PBS and fixed with 4% paraformaldehyde for 40 min-60 min at room temperature, rinsed three times with PBS, and stained with Oil Red O working solution [2:3 Oil Red O in isopropanol and distilled water] for 30min. Excess stain was removed by washing with PBS and observed under an inverted fluorescent microscope.

Then cultured cells were fixed with 4% paraformaldehyde. Cells were then rinsed with PBS and incubated in Oil Red O working solution for 30 min. When the staining solution was removed, Oil Red O was extracted from the cells using 100% isopropanol, and measured at 540 nm using an Ultraviolet Spectrophotometer 1000, and its concentration was determined against a cell-free well with the same treatment [10].

2.4. Deep sequencing analysis

Triplicate cells RNA after 24 h and 48 h Ad-FABP4 treatment were prepared for RNA-Seq analysis separately, Ad-NC group were used as reference. RNA sequencing were performed by Illumina HiSeqTM PE125 in Beijing Novogene company. In addition, differentially expressed genes were screened and were classified into categories by cellular component, molecular function and biological process using GO annotation. A hypergeometric test was applied to map all differentially expressed genes to terms in the GO database and search for significantly enriched GO terms. Pathway analysis were carried out based on the KEGG biological pathway database. The calculated P values were corrected using the Bonferroni correction, using the corrected $|\log_2(\text{Fold Change})| > 1$, q-value < 0.001 as the significance threshold. (The list of differentially expressed genes have shown in attachments, " + " represents upregulated and " - " means downregulated.)

2.5. Quantitation of mRNA expression using real-time PCR

Total RNA was extracted from cultured cells using a Total RNA Kit I(OMEGA). First strand cDNA synthesis was performed with 1 μg of total RNA using Prime Script RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The expression levels of partial differentially expressed genes identified by RNA-Seq were verified by real-time PCR at 0 h, 24 h, 48 h, 96 h after treatment with Ad-FABP4. The primers sequences of these genes are shown (Table 1). Each RT reaction served as template in a 20 μL PCR reaction that contained SYBR Premix Ex Taq™II (TaKaRa, Dalian, China). The reaction mixtures were incubated in the ABI 7500. Analytical data were normalized to the mRNA expression level of endogenous control B2M and GAPDH (Using software NormFinder, GeNorm and Bestkeeper select the most stable control gene in this experiment) [17]. Relative expression levels of objective mRNA were calculated using the $2^{-\triangle\triangle Ct}$ method.

2.6. Statistical analysis

All gene expression data were presented as the mean ± SD ($n = 3$) and one-way ANOVA analysis was conducted using T test for multiple comparisons using the SPSS 17.0 program. Probability (P) value less than 0.05 and P less than 0.01 were considered to be statistically significant or very significant in all statistical analyses, respectively.

Table 1
Primer sequences for RT-PCR.

Gene name	Accession numbers	Forward primer (5′>3′)	Reversed primer (5′>3′)	Fragments size (bp)
DPT	NM_001045903	CAGTACCTGTGAGGAGGTTTAG	CTAAGGCAGAAGGTCTGTTCTC	133
APOD	NM_001076301	GAGAGCTGATGGAACTGTGAA	TGACGGCATGAACCAGAAA	103
SLC4A11	HGNC:16438	CGATTGTGAACGTGAAGGAGA	TGTAGAGGAAGAGGCCATAGAG	132
TTR	NM_173967	CTTCCGTCTGTTCCTCCTTTG	CTTGACCATCAGAGGACACTTG	97
CHI3L1	NM_001080219	AACCTGAAGACCCTCCTATCT	TGGCACCGACTTGATGAAA	108
SFRP1	NM_174460	ACAAACTCACCACCACACTC	GCACTCCCTTGGGCTATTT	91
C3	NM_001040469	TCAAGGTGAGGGTGGAATTG	GACCTGGCTGGGATTGTTATAG	97
PI16	NM_001024487	CCAGAAGAGGCTCAGGATTTG	GTGGAGGTTCTGTTGCTAAGG	118
EGR1	NM_001045875	CCACCTCATCTTCAGCATCAT	CAGGGAAGATGTCAGTGTTAGG	147
STC2	NM_001192745	GCTACCTGAAGCATGATCTGT	TAGGTCCACGTAGGGTTCAT	107
CXCL12	NM_001113174	ATGCCCTTGCCGATTCTT	CGATCTGAAGGGAGCAGTTT	92
ANGPTL4	NM_001076483	GAGAGAGAGAGAGACGGAAAGA	CACACACACACACACCAAAC	100
B2M	NM_173893	AGCAAGGACTGGTCTTTCTAC	GGTCTCGATCCCACTTAACTATC	118
GAPDH	NM_001034034	GATGCTGGTGCTGAGTATGT	GCAGAAGGTGCAGAGATGAT	113

3. Results

3.1. Isolation and characterization of cow MDSCs

The PP6 cells showed a more rounded shape similar to stem cells. To further demonstrate that the PP6 cells were MDSCs, we investigated the expression of Sca1, CD34, Pax3/Pax7 and desmin which were well-defined marker for putative MDSCs in PP6 cells.

Immunocytochemistry showed that the 95.44 ± 1.23% of the PP6 cells were Sca1 positive, 96.37 ± 2.56% were desmin positive, 93.48 ± 1.28% were CD34 positive, and 94.65 ± 2.21% were Pax3/Pax7 positive which indicated most of the PP6 were muscle-derived (Fig. 1). These results suggested that the PP6 cells isolated from cattle skeletal muscles were highly purified MDSCs.

3.2. Effects of ectopic expression of FABP4 gene on MDSCs

To determine whether ectopic expression of FABP4 could stimulate transdifferentiation of MDSCs into adipocyte-like cells, the changes in MDSCs morphology were investigated over time after gene FABP4 were expressed ectopically. Oil Red O staining results indicated the bovine primary treated with FABP4 exhibited an adipocyte phenotype and produced small lipid droplets gradually at 24 h, compared with MDSCs cultured Ad-NC and Control group (Fig. 2A–C). There were no significant differences between Ad-FABP4 and Ad-NC in cell morphology and the accumulation of lipid droplets in cells amount was also not significant ($P > 0.05$). But at infected 48 h, the cells showed obvious variations, cell rounding and many cells aggregate together, and there are more cell lipid

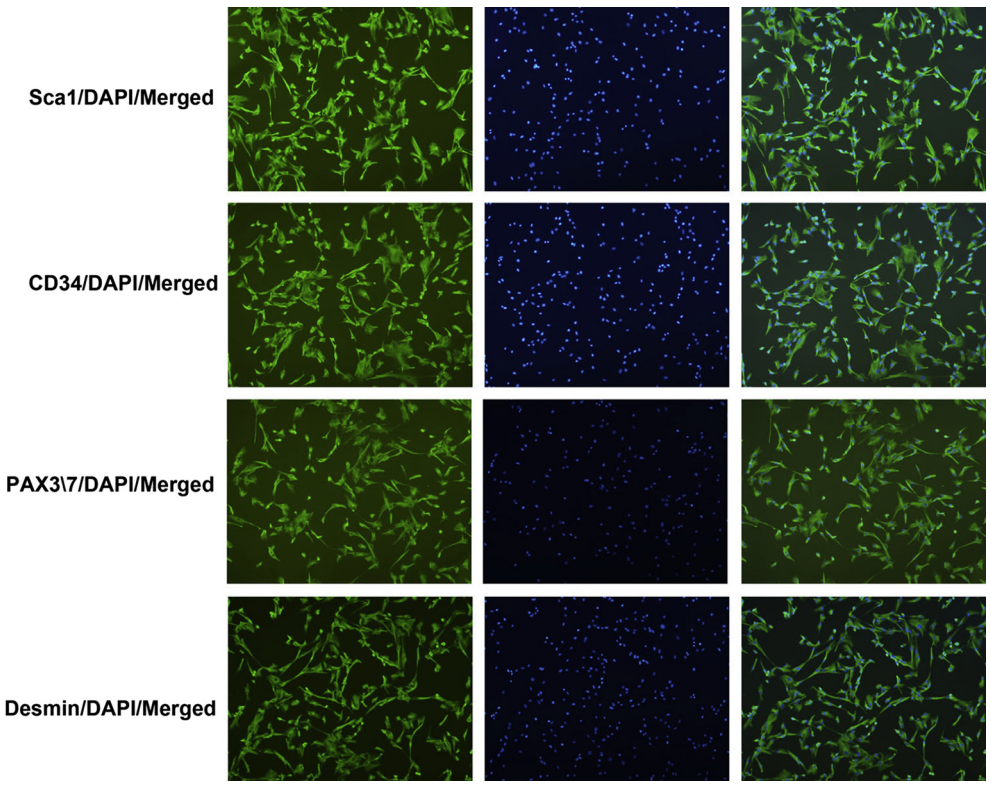

Fig. 1. Isolation and Characterization of cattle MDSCs. Identification of muscle satellite cell specific markers Sca1, desmin, CD34 and Pax3/Pax7 by immunofluorescence (data are mean % cells± S.E.M.). All photomicrographs were taken by an Olympus inverted research microscope (100× magnification) All experiments were repeated at least three times in triplicates.

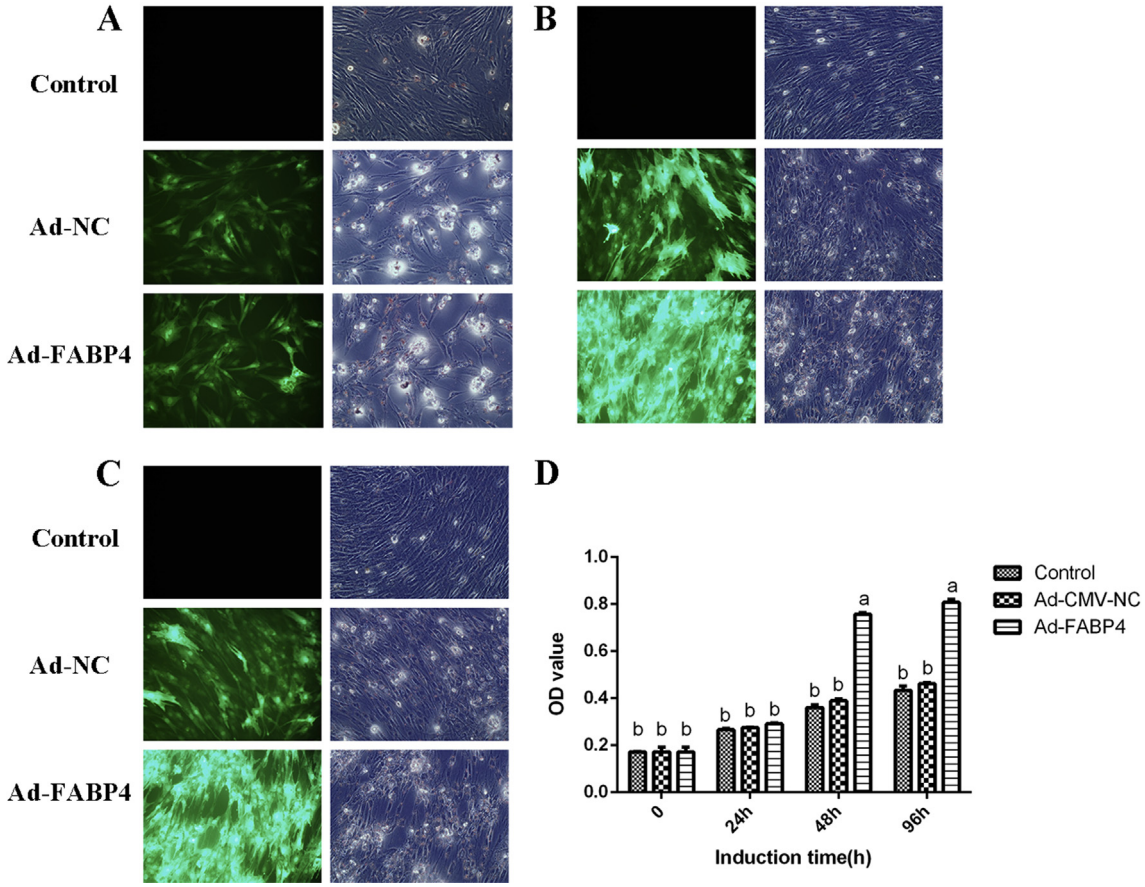

Fig. 2. Lipid accumulation was assessed by Oil Red O staining in cells of Control, Ad-FABP4 and Ad-NC group. A–C: Cell micrographs were taken on ectopical expression of FABP4 gene in MDSCs 24 h, 48 h and 96 h. (× 200 magnification) D: Oil Red O staining extraction infected FABP4 0 h, 24 h, 48 h and 96 h. Intercellular lipid accumulation was determined average values of three replicates are given with mean ± SD (n = 3). a-b means the significant differences between treatment and control groups ($P < 0.05$).

droplets. By measuring the accumulation of lipid droplets, it can be found after 48 h inducing by ectopic expression of FABP4, the formation of lipid droplets were significantly higher than group Ad-NC($P < 0.05$), it also significantly higher than the group with 24 h inducing, but there were no significant differences between 48 h and 96 h in Ad-FABP4 group ($P > 0.05$) (Fig. 2D).

3.3. Deep sequencing analysis of the transdifferentiation process

In order to reveal the mechanisms how FABP4 acts in trans-differentiating MDSCs into adipocyte-like cells, RNA sequencing was performed to profile the gene expressed in MDSCs during the transdifferentiation process. FABP4 will show priority of affecting genes interacted with it directly, and we believe these genes are the core genes co-acted with FABP4 and promote adipogenesis in MDSCs. So we examined the gene expression profiles at the very early stage (24 h, 48 h) of inducing. 23 genes were identified as differentially expressed between the Ad-FABP4 group and Ad-NC group after infected 24 h. Among these genes, 21 genes showed a significant upregulation. The rest two, mir-2904-3 and mir-2904-1 was down-regulated. Nevertheless, when infected for 48 h, there are 256 differentially expressed genes between Ad-FABP4 and Ad-NC group, 110 genes showed an upregulation (supl.1,2). The differentially expressed genes were classified into categories by cellular component, molecular function and biological process using GO annotation (supl.3). Most of the genes are correlated to cellular component. According to the KEGG analysis, after infected 24 h, most of the differentially expressed genes were in Complement and coagulation cascades and Systemic lupus erythematosus pathway are associated with cellular immunity and inhibition of cell proliferation and differentiation, and Hematopoietic cell lineage and Complement and coagulation cascades after infected 48 h. However, many genes in many signaling pathways are associated with fat formation, and PPAR signaling pathway and PI3K-Akt signaling pathway were also expressed (Fig. 3).

3.4. Identification and validation of the differentially expressed genes during transdifferentiation via qPCR

To confirm the result of RNA-seq, we used qPCR to validate the expression levels change of treated with Ad-FABP4 in MDSCs. B2M and GAPDH were used to normalize the gene expression (Fig. 4A–C), all the differentially expressed genes were confirmed by the real-time PCR. All of them have the significant differences ($P < 0.01$) between Ad-FABP4 and Ad-NC.

4. Discussion

In recent years, there has been more profound understanding behind the regulation of adipocyte and muscle cell differentiation, growth and development at the molecular level. In vivo, muscle cells and fat cells are mixed distributed. Both cell types can influence proliferation and differentiation processes by autocrine or paracrine signaling, and ultimately affect the quality of beef [18]. Previous research works on meat quality traits are carried out based on intramuscular content or intramuscular adipocyte

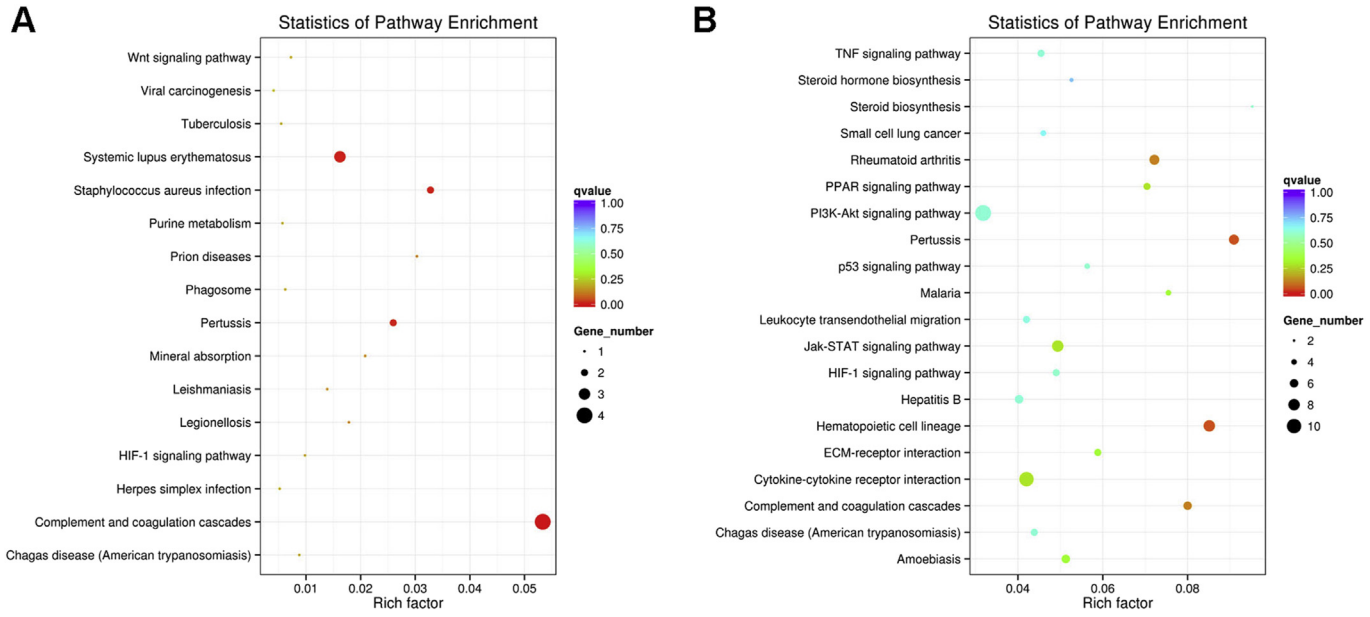

Fig. 3. Deep sequencing analysis of the transdifferentiation process. The functional pathway of differentially expressed genes: A:Ad-FABP4(24 h) VS Ad-NC(24 h) B: Ad-FABP4(48 h) VS Ad-NC(48 h). U sing the corrected |log2(FoldChange)| > 1, qvalue < 0.001 as the significance threshold.

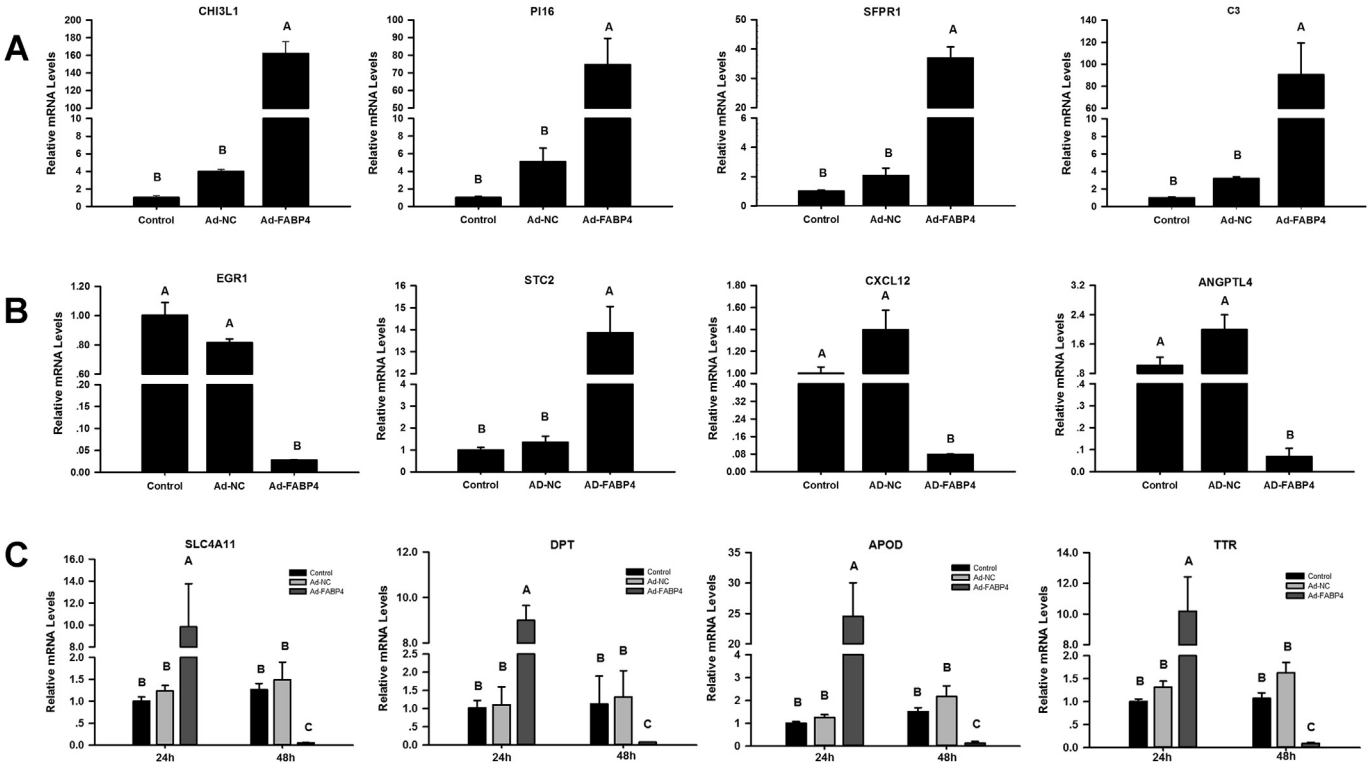

Fig. 4. Deep sequencing results confirmed by qRT-PCR. The measured RNA expression levels were normalized to B2M and GAPDH. Average values of three replicates are given with the mean ± SD (n = 3). A-C:The expression levels of four differentially expressed genes after infected 24 h, 48 h and both two time points determined by real-time PCR. A–C means significant differences between and control, Ad-FABP4 and Ad-NC groups (P < 0.01).

content. Being differentiated from mesodermal derived multipotent stem cells like other fat depositions, it is suggested that intramuscular adipocytes might also be transdifferentiated from the myoblasts in muscle under certain stimulus [19]. But very little is known about this event, especially the regulation and molecular mechanisms of the transdifferentiation.

Many studies have confirmed some of the adipogenic transcription factors such as C/EBPα, PPARγ and SREBP-1 as key regulators that control adipocyte differentiation by inducing fibroblasts and myoblasts transdifferentiation to adipocyte-like cells [9,10,20]. As a downstream gene of adipocyte differentiation pathway, FABP4 was reported to be crucial in the formation of fat, and it was initially

identified as one of the candidate genes that affect the beef tenderness and intramuscular fat content [12]. However, findings from our previous study showed that FABP4 does not affect the differentiation of bovine preadipocytes and cannot change the expression of upstream genes [15]. In this study, we address the key question of whether FABP4 play a key role in lipid synthesis by ectopical expression of FABP4 in bovine MDSCs using adenovirus vector, and activation of adipogenesis were induced in MDSCs. To get a better understanding of the mechanisms that lead to the terminal transdifferentiation of MDSCs, we concentrated our efforts on the investigation of genes that were significantly regulated during this process. There was no significant difference on the lipid droplets were found between inducing and control group at 24 h. On the other hand, lipid droplets observed on large number of cells after 48 h infection were significantly higher in comparison to two other reference groups. Comparing the lipid droplets amount between 48 h and 96 h inducement however show no significant difference.

GO analysis of differentially expressed genes reveals upregulation of genes that are mainly involved in inhibition of cell proliferation and differentiation, indicating a key regulation in cancer suppression. To confirm the RNA-seq result, we validated the gene expression level of selected candidate genes with qPCR. Findings from the present study showed that ectopical expression of FABP4 promote the expression of genes that inhibit myoblast differentiation like Secreted Frizzled-related proteins 1(SFRP1). Gene Sfrp1 have been previously reported to be extracellular regulators of Wnt signal and play important roles in developmental and oncogenic processes. It is also known to regulate the inhibition of myoblast differentiation, further confirming our results [21]. After 48 h infection, cancer suppressor genes like stannio calcin 2(STC2) and EGR1 are also significantly differentially expressed between Ad-FABP4 and Ad-NC. While STC2 was up-regulated, EGR1 was found to be down-regulated in transdifferentiation process. Previous studies have shown that EGR1 is a nuclear protein and functions as a transcriptional regulator. The products of target genes that EGR1 activates are required for differentiation and mitogenesis [22]. Constitutive ectopical expression of human STC2 in mice have been reported to effect pre-and postnatal growth restriction, reduced bone and skeletal muscle growth, and organomegaly [23]. Therefore, we believed that down-regulation of EGR1 through ectopical expression of FABP4 can suppress the muscle cell differentiation while the up-regulation of STC2 reduce the growth of skeletal muscle cells. To sum it up, findings from our study suggest that inhibition of myoblast differentiation was achieved through regulation of genes that are related to the skeletal muscle cells proliferation and differentiation at 24 h and 48 h transdifferentiation. In terms of lipid droplets formation, there was significant difference detected between 24 h and 48 h of FABP4 ectopical expression. This could be due to the fact that at 48 h FABP4 ectopical expression, many differentially expressed genes like interleukin-6 (IL6) and Angiopoietin-like 4 (ANGPTL4) related to adipogenesis have been activated. According to previous studies, reducing fat mass in obesity decreases both circulating leptin and IL6 level [24]. Although ANGPTL4 has been reported to be highly expressed in liver and adipose tissue, it is an inhibitor of circulating lipoprotein lipase (LPL) and a downstream target gene of PPAR [25]. Up-regulation of IL6 and down-regulation of ANGPTL4 in our result suggested that ectopical expression of FABP4 result in activation of IL6 expression and inhibition of ANGPTL4 expression, which lead to promotion of transdifferentiation of adipocyte-like cells. The RT-PCR results of the present study were in agreement with those of the gene chips. Combine with previous studies, we predict that Ectopical expression FABP4 may directly influence the expression level of genes related to inhibition of myoblast differentiation, and activitation of adipogenesis. However, it does not have effect on the gene expression level in upstream genes like C/EBPα, PPARγ and SREBP-1.

Ectopical expression FABP4 gene was found to activate pathways associated with suppression of cancer, cell proliferation and differentiation directly when muscle satellite cells were induced to differentiate into adipocyte-like cells. This was different from the set of genes that were reportedly triggered when preadipocytes were differentiated into adipocyte cells [26]. High reads were detected on pathways associated with complement and coagulation cascades signaling, systemic lupus erythematosus, and hematopoietic cell lineage. Contrary to our findings, other studies previously reported detection in pathways associated with cellular immunity, blood coagulation, and inhibition of cell proliferation and differentiation [27–29]. Additionally, PPAR pathway and PI3K–Akt pathway has been implicated in the regulation of expression of lipid metabolic genes and reported to plays a central role in fat tissue function and energy metabolism [30]. The evidence success of transdifferentiation had been proved through RNA-seq and QPCR. Specific regulatory approach needs further study. Although the function of many genes in the transdifferentiation remain unknown, their consistent expression pattern and high conservation indicate that they are also likely to play roles in the development of bovine muscle tissues.

In conclusion, FABP4 can induce bovine Muscle-Derived Stem Cells transdifferentiation into adipocyte-like cells. 23 genes were initially affected by FABP4 ectopical expression. These genes should be the core genes interacted directly with FABP4 during the process of transdifferentiation, An Assumed regulation mechanism of FABP4 regulate MDSCs transdifferentiation was shown in supl.4.

Conflicts of interest

We declare that we have no conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.11.067.

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